Chapter

In vitro Cell-Based Assays for Potency Testing of Anti-TNF-α Biological Drugs

Sara Žigon-Branc, Ariana Barlič and Matjaž Jeras

Abstract

Human cell-based assays for *in vitro* testing of drugs in preclinical and research studies, as well as in clinical practice, are gaining greater importance especially in view of personalized medicine, which is tailored to the individual needs and benefits of a patient. This chapter begins with an overview of contemporary cell-based assays, routinely used for a comparative *in vitro* potency testing of anti-TNF-α innovator biologics and their biosimilars. In sequel, based on the results of our original work, we will further discuss the establishment and use of 2D normal and osteoarthritic primary chondrocyte monolayer cultures and 3D microspheroidal articular cartilage tissues, prepared in hanging drops from osteoarthritic chondrocytes and chondrogenically differentiated mesenchymal stem cells. Both 2D and 3D cultures will be presented as models for assessing the neutralizing potency of the three well-known anti-TNF-α biological drugs: adalimumab, etanercept, and infliximab.

Keywords: *in vitro* cell-based assays, anti-TNF-α biologics, human articular chondrocytes, mesenchymal stem cells, 2D monolayer cultures, 3D cell cultures, gene expression

1. Introduction

Following the discovery and characterization of tumor necrosis factor (TNF) in the mid-1980s, this pleiotropic proinflammatory cytokine continues to be the focus of numerous studies and represents an important therapeutic target [1, 2]. The venue of anti-TNF biological drugs has revolutionized treatment of autoimmune and inflammatory diseases like rheumatoid arthritis, juvenile idiopathic arthritis, ankylosing spondylitis, psoriasis, Crohn's disease, ulcerative colitis, and others [2]. Although expensive, biological drugs (biologics) at the moment represent the best-selling group of pharmaceuticals. Nowadays, following the expiry of originators patents, a plethora of less expensive biosimilar drugs (biosimilars) are available to patients. In order to confirm the biocomparability of original and biosimilar products and to prove their quality, safety, and efficacy, the use of reliable and standardized bioassays relevant in assessing their modes of action is of crucial importance.

In this chapter, after a short introductory review of TNF biology, anti-TNF biological drugs and their mechanisms of action, we will present a selection of *in vitro* cell-based tests used either for general or personalized potency testing of anti-TNF biologics and their biosimilars.

2. A short overview of TNF biology

TNF is produced in various cell types, mainly immune cells such as monocytes and macrophages, microglia, neutrophils, natural killer cells (NK), T lymphocytes, and also in neuronal cells, keratinocytes, and fibroblasts [2, 3]. The cytokine exists in two biologically active forms. The first being a transmembrane protein (tmTNF), which can be cleaved by the metalloproteinase TNF-α-converting enzyme (TACE) (also known as disintegrin and metalloproteinase domain-containing protein 17 (ADAM17)) into its second form, a homotrimeric soluble TNF (sTNF) [2].

There are two TNF-binding homotrimeric transmembrane receptors, namely the TNF receptor 1 (TNFR1 or CD120a) and the TNF receptor 2 (TNFR2 or CD120b) [2]. While the TNFR1 is constitutively expressed on a vast majority of nucleated cells, the TNFR2 expression is inducible and tightly regulated, preferentially on endothelial, hematopoietic, neural, and immune cells [2, 4]. TNFR2 is also expressed on tumor cells where it is supposed to function as a tumor oncogene [5, 6].

Interestingly, tmTNF can induce signals in a bipolar way, as it acts as a ligand of both receptor types and as a receptor itself in cell-to-cell contacts [2, 4]. This means that tmTNF-α-expressing cells transmit signals to cells bearing TNFR1 and/or TNFR2. This phenomenon is called "outside-to-inside" or "reverse signaling," the function of which has not been completely clarified yet [2, 4]. The receptor function of tmTNF has been demonstrated in human monocytes, macrophages, NK cells, and T lymphocytes [4].

While TNFR1 is activated by both tmTNF and sTNF, TNFR2 can only be triggered by tmTNF. Both types of membrane-bound receptors are prone to TACE cleavage, resulting in fragments termed soluble TNF receptors (sTNFR) [2]. In turn, sTNFR may contribute to the regulation of cellular TNF responses by capturing and neutralizing circulating TNF (intrinsic TNF inhibitors). Additionally, due to increased receptor shedding, the number of functional signaling membrane TNFRs decreases. Consequently, this leads to a state of transient TNF desensitization [2].

3. Anti-TNF biological drugs and their mechanisms of action

Among currently available Food and Drug Administration (FDA)- and European Medicines Agency (EMA)-approved originator and biosimilar anti-TNF drugs, there are three full-length monoclonal antibodies (mAbs); these are infliximab (IFX), a chimeric mouse/human mAb (Remicade® and its biosimilars: Remsima®, Inflectra®, Flixabi®, Ixifi®, Renflexis®, and Zessly®), adalimumab (ADA), a fully humanized mAb (Humira® and its biosimilars: Cyltezo®, Imraldi®, Amgevita®, Solymbic®, Hyrimoz®, Hulio®, Halimatoz®, and Heyifa®), and golimumab, another fully humanized mAb (Simponi®) (**Figure 1**) [2, 4]. The additional two anti-TNF biological drugs, which are not mAbs, are etanercept (ETA) (Enbrel® and its biosimilars: Erelzi® and Benepali®), a fusion protein consisting of two extracellular parts of the human TNFR2 and the Fc portion of human IgG1, and certolizumab pegol (Cimzia®) composed of a human Fab' fragment, covalently attached to two cross-linked 20 kDa polyethylene glycol chains (**Figure 1**) [2, 4].

Although all anti-TNF biologics neutralize the same target (sTNF and tmTNF), they are not equally effective in treatment of certain inflammatory pathologies, for example, Crohn's disease. This is due to differences in their characteristics (structure and binding affinities) and mechanisms of action (**Figure 1**) [2, 4]. Besides all of them being efficacious in neutralizing both forms of TNF, infliximab additionally induces "outside-to-inside" signaling via binding to tmTNF, thereby triggering apoptosis of tmTNF-expressing immune cells [2]. Being full-length mAbs,

chondrogenic tissue model which, combined with the qRT-PCR readout method, can be used for preclinical or patient-specific potency assessment of anti-TNF-α and anti-interleukin-1β biological drugs (anti-IL-1β) [26]. For establishing this model, we used human OACs and chondrogenically differentiated MSCs.

As already stated, OACs represent an attractive source of cells for cell-based models as besides being rather easily accessible and free of ethical concerns, they are also genetically stable during their long-term *in vitro* expansion [36, 37]. Reports show that MSCs isolated from bone marrow of OA patients are capable of producing hyaline cartilage suitable for tissue repair. MSCs obtained from OA and RA patients possess similar chondrogenic potential as those from healthy individuals [38–41]. Therefore, we used paired samples of MSCs and OACs from two donors and a set of genetically mismatched biological samples of patient's OACs and commercially available MSCs. The paired cell sampling approach allowed us to reduce the high patient-to-patient variability, which influences the chondrogenic potential of both OACs and MSCs [42].

Among the numerous commercially available 3D cell culture systems, we have chosen Perfecta 3D® scaffolds (3D Biomatrix Inc., USA) to create tissues in hanging drops. Generation of scaffold-free spheroids of micrometric dimensions (microspheroids) by gravity-enforced self-assembly in hanging drops allows cell aggregation and tissue formation in a natural manner, without interference from the scaffold material [19, 32]. This technique has important advantages, especially the drop size control and consequent uniformity of formed microspheroids. Moreover, it is compatible with automated liquid handling systems, a prerequisite for high-throughput screening in drug discovery. The microspheroid formation in hanging drops mimics the condensation process of MSCs, which is one of the earliest phases of *in vivo* cartilage development [32].

Isolated OACs were first expanded in 2D monolayer cultures and then, from passage 2 and on, 10,000 cells were transferred into each hanging drop. In this way, the loss of chondrogenic phenotype of OACs in 2D was restored in 3D conditions, as already reported [30, 43]. Similarly as in our previously described 2D primary chondrocyte model, the TNF-α neutralizing efficiencies of ADA (Humira®, Abbott Laboratories, USA), ETA (Enbrel®, Immunex Corp., USA), IFX (Remicade®, Janssen Biotech, USA), and the anti-IL-1β drug anakinra (ANA; Kineret®, Swedish Orphan Biovitrum AB, Sweden) were assessed with both cell types by determining the extent of downregulation of six selected genes (*IL6*, *IL8*, *MCP1*, *MMP1*, *MMP13*, and *VCAM1*) [27, 28]. Gene expression was determined after a 24 h incubation of microspheroids in a medium supplemented with 1 ng/mL of an appropriate inflammatory cytokine (rhTNF-α or rhIL-1β; both from PeproTech, USA) or working macrophage conditioned medium (MCM) solution, combined with 1 μg/mL of each individual biological drug tested (**Figure 4**).

According to our criteria, Log_2 RQ ≥1 and ≤ −1, TNF-α significantly upregulated the expression of *IL6*, *IL8*, *MCP1*, *MMP1*, *MMP13*, and *VCAM1* genes in the 3D microspheroidal model as well (**Figure 4a**). The same was true when IL-1β or MCM was added to microspheroids. MCM was obtained from cell cultures of the human monocytic cell line THP-1 (ATCC, USA) and represented a rich source of inflammatory cytokines with 0.05 ng/mL TNF-α and 0.45 ng/mL IL-1β, and numerous other growth factors. In terms of influencing gene expression, IL-1β was the most potent inflammation inducer, followed by MCM and then TNF-α. The inflammatory process triggered by each of these three inducers could always be reversed by ADA, IFX, or ETA, as well as ANA (**Figure 4a**). When inflammation was triggered by TNF-α, all tested anti-TNF-α biologics extraordinarily suppressed the expression of monitored genes, sometimes even reaching their constitutively expressed levels (log2 RQ = 0). Similarly, in the presence of IL-1β, ANA markedly reversed the

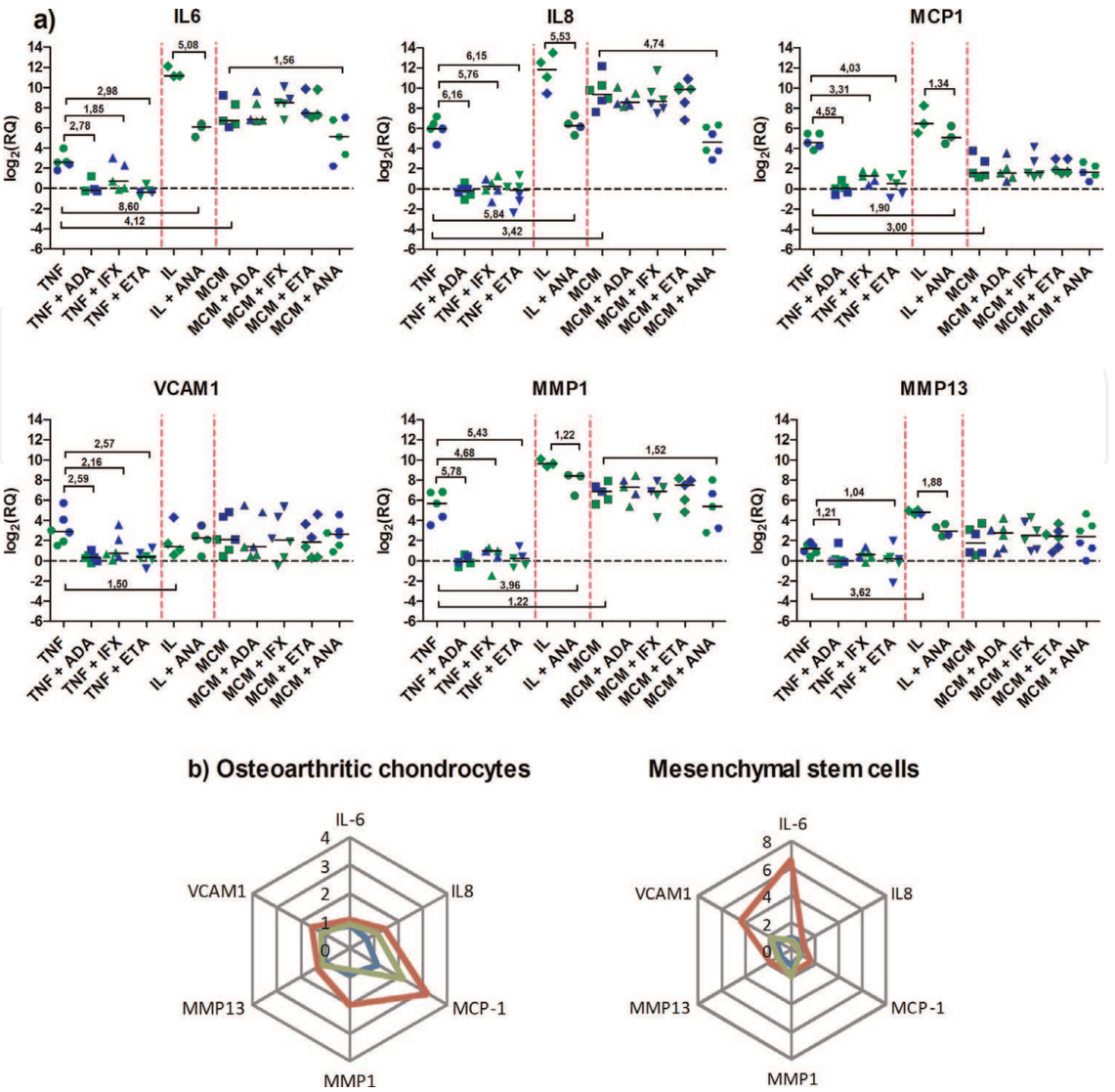

Figure 4.
(a) Gene expression profiles following the addition of inflammatory mediators TNF-α, IL-1β, or MCM working solution and anti-inflammatory biological drugs ADA, IFX, ETA, and ANA. Blue and green dots represent values obtained in microspheroidal chondral tissues made of MSCs and OACs (three donors), respectively. Statistically significant changes, that is, Log2 RQ ≥1 and ≤ −1 are outlined with median values for all groups. (b) Radar graphs representing anti-TNF-α neutralization efficacies of ADA (blue), IFX (red), and ETA (green). Mean RQ values of three biological samples are shown for OAC- and MSC-derived microspheroids. Value 0 in the center of each radar graph represents total inhibition of gene expression. Original figure used with authors' permission under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/) [26].

inflammation process in microspheroids. However, when microspheroids were incubated with MCM, none of the three tested anti-TNF-α drugs were successful in diminishing its inflammatory effect. Conversely, ANA could downregulate the expression of *IL6*, *IL8*, and *MMP1* genes. The described changes at the gene level were accompanied by significant differences in the expression of IL6, IL8, and MCP1 proteins, detected in supernatants of microspheroid cultures, 24 h after their incubation with a given inflammatory agent ± selected anti-inflammatory biological drug [26]. Moreover, these results were additionally supported by the amount of glycosaminoglycans present in chondral spheroids composed of 100,000 OACs treated with various combinations of a particular inflammatory agent ± a given anti-inflammatory biologic, for a period of 3 weeks.

When microspheroids were incubated with MCM, a superior anti-IL-1β neutralization capacity of ANA compared to the three tested anti-TNF-α biologics was observed. This difference was probably due to the fact that MCM contained a much

higher concentration of IL-1β (0.45 ng/mL) than TNF-α (0.05 ng/mL). Nevertheless, these concentrations of both cytokines are much higher than those measured in synovial fluids of OA and RA patients (0.028 ng/mL TNF-α and 0.1 ng/mL IL-1β) [44]. Although MCM proved to be an excellent *in vitro* inducer of inflammation, its use for potency testing of anti-inflammatory biologicals targeting a specific cytokine is questionable. In fact, from the multiple synergistic proinflammatory effects evoked by different biogenic factors present in MCM, it is very hard to define the potency of a biological targeting a single inflammatory factor.

In our 3D microspheroidal rhTNF-α-induced inflammation model, the neutralization capacity of ADA was superior over that of ETA and the even weaker IFX (**Figure 4b**). Similar results were obtained with both microspheroids, regardless of whether they were made of OACs or chondrogenically differentiated MSCs. The observed differences in neutralizing efficiencies of ADA, ETA, and IFX can be attributed to differences in their molecular structures and sTNF-α-binding affinities [45]. The superior anti-TNF-α efficacy of ADA over ETA and IFX has already been reported together with data, showing that the sTNF-α-binding affinity of ADA is higher for ADA (Kd = 7.05×10^{-11}) than ETA (Kd = 2.35×10^{-11}) and IFX (Kd = 1.17×10^{-10}) [46–48]. However, according to our criterion, a particular biologic would be statistically more efficient than the compared one if it would cause a ≥2-fold decrease in a selected gene expression. This was not the case in any of our 3D microspheroidal model experiments. Consequently, we assumed that the observed differences in TNF-α neutralizing potency of ADA, ETA, and IFX were comparable (**Figure 4b**). Interestingly, although we showed in our 2D OACs model that ETA was significantly more efficient than IFX, the same kind of experiments carried out in a 3D microspheroidal model did not confirm this finding [26, 27]. We assume that compared to the 2D model, the diffusion of tested biologics in our 3D microspheroidal model was much slower and limited. Undoubtedly, the 3D model better resembles *in vivo* conditions and therefore has a higher relevance. Thus, we concluded that 2D cell culture models may be useful for obtaining preliminary data regarding the anti-inflammatory effects of a particular biological drug, while 3D microtissue models enable more relevant insights in drug-tissue interactions and possible outcomes *in vivo*. The results obtained with our 3D microspheroidal model are also supported by the outcomes of clinical studies conducted on patients with RA, where the efficacies of anti-TNF-α biologics proved to be comparable [49].

We found that OACs and chondrogenically differentiated MSCs are suitable sources for hanging drop chondral 3D microspheroid cultures formation, which are useful for the assessment of neutralization potencies of anti-inflammatory biologics [26]. Although the use of these two types of microspheroids resulted in different gene expression profiles following their incubation with tested combinations of rhTNF-α, and each of the three tested anti-TNF-α biological drugs (**Figure 4b**), these differences were rather small. Therefore, we concluded that MSCs can be used as an alternative and probably even more accessible cell source for *in vitro* testing of neutralization potency of anti-TNF-α biologics. The main advantages of our 3D model are the use of small amounts of human cells and cytokines, personalized testing approach, and the possibility of automation. In addition, the presented approach can also be used as a platform for testing other anti-inflammatory biologics with different mechanisms of action, as shown for ANA, the antagonist of IL-1β.

6. Conclusion

Cell-based assays are complex analytical tools, susceptible to multiple variables that are virtually impossible to control. Therefore, they have to be precise, reliable,

and well standardized so that the results are reproducible and can be compared among different laboratories. When used for drug potency testing, such assays usually rely on the use of reference standards. Recently, the WHO has prepared two international standards for the two anti-TNF-α biologics, etanercept and infliximab. These have been tested by several laboratories within an international collaborative study using a number of different cell-based assays [12, 13]. In this chapter, we have presented an overview of the most routinely used tests for potency testing of anti-TNF-α biologics, which measure *in vitro* responses of nonmanipulated or genetically engineered human and animal cell lines, with various readout systems.

Nowadays, with an expanding personal medicine approach, laboratory assay-guided pharmacotherapeutical strategies are becoming more and more important. In order to obtain relevant data on drug potencies for a particular patient, these kinds of tests should be based on the patient's own, that is, autologous primary cells, as these can significantly reduce costs and enable safer and more effective therapies. Therefore, we dedicated a part of this chapter to our experience in establishing *in vitro* 2D monolayer cultures consisting of normal and OA chondrocytes and 3D microspheroidal chondral tissues, formed from OACs or chondrogenically differentiated bone-marrow-derived MSCs, and their use for testing anti-TNF-α efficacy of adalimumab, etanercept, and infliximab. The qRT-PCR technique was applied as a readout system for assessing differences in selected gene expressions. The obtained data led us to the establishment of an original statistical method, which was used for the evaluation and comparison of results.

Acknowledgements

The authors wish to express their gratitude to Prof. Andrej Blejec, Dr. Miomir Knežević, Asst. Prof. Nevenka Kregar Velikonja, and Prof. Gordana Vunjak-Novakovic for their contribution to our common, published original research work, partly presented in this chapter. We are also grateful to Rosmarijn E. Vandenbroucke for approving the use of a picture presented in **Figure 1** and to Andrej Branc for proofreading the manuscript.

Conflict of interest

The authors declared that no competing interests exist.

Appendices and nomenclature

2D	two-dimensional
3D	three-dimensional
ADA	adalimumab
ANA	anakinra
ETA	etanercept
IFX	infliximab
IL-1β	interleukin 1β
MCM	macrophage conditioned medium
MSCs	mesenchymal stem cells
NCs	normal human articular chondrocytes
OACs	osteoarthritic human articular chondrocytes
RQ	relative quantity of gene expression

rhTNF-α	recombinant human tumor necrosis factor α
sTNF-α	soluble form of tumor necrosis factor α
tmTNF-α	transmembrane form of tumor necrosis factor α

Author details

Sara Žigon-Branc[1†], Ariana Barlič[2†] and Matjaž Jeras[3*]

1 Institute of Materials Science and Technology, Technische Universität Wien, Vienna, Austria

2 Educell Cell Therapy Service Ltd., Trzin, Slovenia

3 Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia

*Address all correspondence to: matjaz.jeras@ffa.uni-lj.si

† These authors contributed equally to this work

References

[1] Aggarwal BB, Gupta SC, Kim JH. Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. Blood. 2012;**119**:651-665. DOI: 10.1182/blood-2011-04-325225

[2] Steeland S, Libert C, Vandenbroucke RE. A new venue of TNF targeting. International Journal of Molecular Sciences. 2018;**19**:1442. DOI: 10.3390/ijms19051442

[3] Yan L, Hu R, Tu S, Cheng W-J, Zheng Q, Wang J-W, et al. Establishment of a cell model for screening antibody drugs against rheumatoid arthritis with ADCC and CDC. International Journal of Clinical and Experimental Medicine. 2015;**8**: 20065-20071

[4] Mitoma H, Horiuchi T, Tsukamoto H, Ueda N. Molecular mechanisms of action of anti-TNF-α agents—Comparison among therapeutic TNF-α antagonists. Cytokine. 2018;**101**:56-63. DOI: 10.1016/j.cyto.2016.08.014

[5] Chen X, Oppenheim JJ. Targeting TNFR2, an immune checkpoint stimulator and oncoprotein, is a promising treatment for cancer. Science Signaling. 2017;**10**:eaal2328. DOI: 10.1126/scisignal.aal2328

[6] Torrey H, Butterworth J, Mera T, Okubo Y, Wang L, Baum D, et al. Targeting TNFR2 with antagonistic antibodies inhibits proliferation of ovarian cancer cells and tumor-associated T_{regs}. Science Signaling. 2017; **10**:eaaf8608. DOI: 10.1126/scisignal.aaf8608

[7] Vos ACW, Wildenberg ME, Duijvestein M, Verhaar AP, van den Brink GR, Hommes DW. Anti-tumor necrosis factor-α antibodies induce regulatory macrophages in an Fc region-dependent manner. Gastroenterology. 2011;**140**:221-230.e3. DOI: 10.1053/j.gastro.2010.10.008

[8] Nguyen DX, Ehrenstein MR. Anti-TNF drives regulatory T cell expansion by paradoxically promoting membrane TNF–TNF-RII binding in rheumatoid arthritis. The Journal of Experimental Medicine. 2016;**213**:1241-1253. DOI: 10.1084/jem.20151255

[9] Nadkarni S, Mauri C, Ehrenstein MR. Anti-TNF-α therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-β. The Journal of Experimental Medicine. 2007;**204**:33-39. DOI: 10.1084/jem.20061531

[10] Shealy DJ, Cai A, Staquet K, Baker A, Lacy ER, Johns L, et al. Characterization of golimumab, a human monoclonal antibody specific for human tumor necrosis factor α. MAbs. 2010;**2**:428-439

[11] Paleolog E. Target effector role of vascular endothelium in the inflammatory response: Insights from the clinical trial of anti-TNF alpha antibody in rheumatoid arthritis. Molecular Pathology. 1997;**50**:225-233

[12] Wadhwa M, Bird C, Dilger P, Rigsby P, Jia H, Gross MEB. Establishment of the first WHO International Standard for etanercept, a TNF receptor II Fc fusion protein: Report of an international collaborative study. Journal of Immunological Methods. 2017;**447**:14-22. DOI: 10.1016/j.jim.2017.03.007

[13] Metcalfe C, Dougall T, Bird C, Rigsby P, Behr-Gross M-E, Wadhwa M, et al. The first World Health Organization International Standard for infliximab products: A step towards maintaining harmonized biological activity. MAbs. 2019;**11**:13-25. DOI: 10.1080/19420862.2018.1532766

[14] Hofmann H-P, Kronthaler U, Fritsch C, Grau R, Müller SO, Mayer R, et al. Characterization and non-clinical assessment of the proposed etanercept biosimilar GP2015 with originator etanercept (Enbrel(®)). Expert Opinion on Biological Therapy. 2016;**16**: 1185-1195. DOI: 10.1080/14712598.2016.1217329

[15] Lallemand C, Kavrochorianou N, Steenholdt C, Bendtzen K, Ainsworth MA, Meritet J-F, et al. Reporter gene assay for the quantification of the activity and neutralizing antibody response to TNFα antagonists. Journal of Immunological Methods. 2011;**373**: 229-239. DOI: 10.1016/j.jim.2011.08.022

[16] Mitoma H, Horiuchi T, Tsukamoto H, Tamimoto Y, Kimoto Y, Uchino A, et al. Mechanisms for cytotoxic effects of anti-tumor necrosis factor agents on transmembrane tumor necrosis factor α-expressing cells: Comparison among infliximab, etanercept, and adalimumab. Arthritis and Rheumatism. 2008;**58**:1248-1257. DOI: 10.1002/art.23447

[17] Ueda N, Tsukamoto H, Mitoma H, Ayano M, Tanaka A, Ohta S, et al. The cytotoxic effects of certolizumab pegol and golimumab mediated by transmembrane tumor necrosis factor α. Inflammatory Bowel Diseases. 2013;**19**: 1224-1231. DOI: 10.1097/MIB.0b013e318280b169

[18] Moore M, Ferguson J, Burns C. Applications of cell-based bioassays measuring the induced expression of endogenous genes. Bioanalysis. 2014;**6**: 1563-1574. DOI: 10.4155/bio.14.98

[19] Rimann M, Graf-Hausner U. Synthetic 3D multicellular systems for drug development. Current Opinion in Biotechnology. 2012;**23**:803-809. DOI: 10.1016/j.copbio.2012.01.011

[20] Baker BM, Chen CS. Deconstructing the third dimension—How 3D culture microenvironments alter cellular cues. Journal of Cell Science. 2012;**125**: 3015-3024. DOI: 10.1242/jcs.079509

[21] Edmondson R, Broglie JJ, Adcock AF, Yang L. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. Assay and Drug Development Technologies. 2014;**12**: 207-218. DOI: 10.1089/adt.2014.573

[22] Duval K, Grover H, Han L-H, Mou Y, Pegoraro AF, Fredberg J, et al. Modeling physiological events in 2D vs. 3D cell culture. Physiology. 2017;**32**:266-277. DOI: 10.1152/physiol.00036.2016

[23] Alépée N. State-of-the-art of 3D cultures (organs-on-a-chip) in safety testing and pathophysiology. ALTEX. 2014;**31**(4):441-477. DOI: 10.14573/altex1406111

[24] Sophia Fox AJ, Bedi A, Rodeo SA. The basic science of articular cartilage: Structure, composition, and function. Sports Health: Multidisciplinary Approach. 2009;**1**:461-468. DOI: 10.1177/1941738109350438

[25] Otero M, Favero M, Dragomir C, Hachem KE, Hashimoto K, Plumb DA, et al. Human chondrocyte cultures as models of cartilage-specific gene regulation. In: Mitry RR, Hughes RD, editors. Human Cell Culture Protocols. Vol. 806. Totowa, NJ: Humana Press; 2012. pp. 301-336

[26] Žigon-Branc S, Barlič A, Knežević M, Jeras M, Vunjak-Novakovic G. Testing the potency of anti-TNF-α and anti-IL-1β drugs using spheroid cultures of human osteoarthritic chondrocytes and donor-matched chondrogenically differentiated mesenchymal stem cells. Biotechnology Progress. 2018;**34**: 1045-1058. DOI: 10.1002/btpr.2629

[27] Žigon-Branc S, Jeras M, Blejec A, Barlič A. Applicability of human osteoarthritic chondrocytes for in vitro

efficacy testing of anti-TNFα drugs. Biologicals. 2017;**45**:96-101. DOI: 10.1016/j.biologicals.2016.09.013

[28] Barlič A, Žigon S, Blejec A, Kregar Velikonja N. Gene expression of cultured human chondrocytes as a model for assessing neutralization efficacy of soluble TNFα by TNFα antagonists. Biologicals. 2015;**43**: 171-180. DOI: 10.1016/j.biologicals.2015.03.001

[29] Burns CJ, Silva MMCG, Gray E, Robinson CJ. Quantitative RT-PCR as an alternative to late-stage bioassays for vascular endothelial growth factor. Journal of Pharmaceutical and Biomedical Analysis. 2008;**47**:460-468. DOI: 10.1016/j.jpba.2008.02.011

[30] Caron MMJ, Emans PJ, Coolsen MME, Voss L, Surtel DAM, Cremers A, et al. Redifferentiation of dedifferentiated human articular chondrocytes: Comparison of 2D and 3D cultures. Osteoarthritis and Cartilage. 2012;**20**:1170-1178. DOI: 10.1016/j.joca.2012.06.016

[31] Mata-Miranda MM, Martinez-Martinez CM, Noriega-Gonzalez JE, Paredes-Gonzalez LE, Vázquez-Zapién GJ. Morphological, genetic and phenotypic comparison between human articular chondrocytes and cultured chondrocytes. Histochemistry and Cell Biology. 2016;**146**(2):183-189. DOI: 10.1007/s00418-016-1437-4

[32] Lehmann M, Martin F, Mannigel K, Kaltschmidt K, Sack U, Anderer U. Three-dimensional scaffold-free fusion culture: The way to enhance chondrogenesis of in vitro propagated human articular chondrocytes. European Journal of Histochemistry. 2013;**57**:31. DOI: 10.4081/ejh.2013.e31

[33] Bhumiratana S, Vunjak-Novakovic G. Engineering physiologically stiff and stratified human cartilage by fusing condensed mesenchymal stem cells. Methods. 2015;**84**:109-114. DOI: 10.1016/j.ymeth.2015.03.016

[34] Baraniak PR, McDevitt TC. Scaffold-free culture of mesenchymal stem cell spheroids in suspension preserves multilineage potential. Cell and Tissue Research. 2012;**347**:701-711. DOI: 10.1007/s00441-011-1215-5

[35] Sart S, Tsai A-C, Li Y, Ma T. Three-dimensional aggregates of mesenchymal stem cells: Cellular mechanisms, biological properties, and applications. Tissue Engineering. Part B, Reviews. 2014;**20**:365-380. DOI: 10.1089/ten.teb.2013.0537

[36] Laganà M, Arrigoni C, Lopa S, Sansone V, Zagra L, Moretti M, et al. Characterization of articular chondrocytes isolated from 211 osteoarthritic patients. Cell and Tissue Banking. 2014;**15**:59-66. DOI: 10.1007/s10561-013-9371-3

[37] Neri S, Mariani E, Cattini L, Facchini A. Long-term in vitro expansion of osteoarthritic human articular chondrocytes do not alter genetic stability: A microsatellite instability analysis. Journal of Cellular Physiology. 2011;**226**:2579-2585. DOI: 10.1002/jcp.22603

[38] Tallheden T, Bengtsson C, Brantsing C, Sjogren-Jansson E, Carlsson L, Peterson L, et al. Proliferation and differentiation potential of chondrocytes from osteoarthritic patients. Arthritis Research & Therapy. 2005;**7**:R560-R568

[39] Kafienah W, Mistry S, Dickinson SC, Sims TJ, Learmonth I, Hollander AP. Three-dimensional cartilage tissue engineering using adult stem cells from osteoarthritis patients. Arthritis and Rheumatism. 2007;**56**:177-187. DOI: 10.1002/art.22285

[40] Jagielski M, Wolf J, Marzahn U, Völker A, Lemke M, Meier C, et al. The

influence of IL-10 and TNFα on chondrogenesis of human mesenchymal stromal cells in three-dimensional cultures. International Journal of Molecular Sciences. 2014;**15**: 15821-15844. DOI: 10.3390/ijms150915821

[41] Dudics V, Kunstár A, Kovács J, Lakatos T, Géher P, Gömör B, et al. Chondrogenic potential of mesenchymal stem cells from patients with rheumatoid arthritis and osteoarthritis: Measurements in a microculture system. Cells, Tissues, Organs. 2009;**189**:307-316. DOI: 10.1159/000140679

[42] Agar G, Blumenstein S, Bar-Ziv Y, Kardosh R, Schrift-Tzadok M, Gal-Levy R, et al. The chondrogenic potential of mesenchymal cells and chondrocytes from osteoarthritic subjects: A comparative analysis. Cartilage. 2011;**2**: 40-49. DOI: 10.1177/1947603510380899

[43] Schulze-Tanzil G, de Souza P, Villegas Castrejon H, John T, Merker H-J, Scheid A, et al. Redifferentiation of dedifferentiated human chondrocytes in high-density cultures. Cell and Tissue Research. 2002;**308**:371-379. DOI: 10.1007/s00441-002-0562-7

[44] Westacott CI, Whicher JT, Barnes IC, Thompson D, Swan AJ, Dieppe PA. Synovial fluid concentration of five different cytokines in rheumatic diseases. Annals of the Rheumatic Diseases. 1990;**49**:676-681

[45] Tracey D, Klareskog L, Sasso EH, Salfeld JG, Tak PP. Tumor necrosis factor antagonist mechanisms of action: A comprehensive review. Pharmacology & Therapeutics. 2008;**117**:244-279. DOI: 10.1016/j.pharmthera.2007.10.001

[46] Hu S, Liang S, Guo H, Zhang D, Li H, Wang X, et al. Comparison of the inhibition mechanisms of adalimumab and infliximab in treating tumor necrosis factor α-associated diseases from a molecular view. The Journal of Biological Chemistry. 2013;**288**: 27059-27067. DOI: 10.1074/jbc.M113.491530

[47] Scallon B, Cai A, Solowski N, Rosenberg A, Song X-Y, Shealy D, et al. Binding and functional comparisons of two types of tumor necrosis factor antagonists. The Journal of Pharmacology and Experimental Therapeutics. 2002;**301**:418-426

[48] Granneman RG, Zhang YM, Noertersheuser PA, Velagapudi RB, Awni WM, Locke CS, et al. Pharmacokinetic/pharmacodynamic (PK/PD) relationships of adalimumab (HUMIRA™, Abbott) in rheumatoid arthritis (RA) patients during phase II/III clinical trials. In: Arthritis and Rheumatism. Vol. 48. Div John Wiley & Sons Inc, 605 Third Ave, New York, NY 10158–0012 USA: Wiley-Liss; 2003. pp. S140-S141

[49] Hyrich KL, Lunt M, Watson KD, Symmons DPM, Silman AJ. British Society for Rheumatology Biologics Register. Outcomes after switching from one anti-tumor necrosis factor alpha agent to a second anti-tumor necrosis factor alpha agent in patients with rheumatoid arthritis: Results from a large UK national cohort study. Arthritis and Rheumatism. 2007;**56**:13-20. DOI: 10.1002/art.22331